Oligonucleotides and Analogues

A Practical Approach

Edited by

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Attachment of a non-radioactive markers

Amino-linked oligonucleotides	
dimethylformamide (analytical grade; free from primary and secondary amines)	
triethylamine, isothiocyanate, or N-hydroxy-succinimide ester derivatives of fluorescein, rhodamine and Texas Red	Molecular Probes Aldrich
N-hydroxysuccinimide ester of biotin	Molecular Probes Pierce
Thiol-linked oligonucleotides	
Silver nitrate	Aldrich
dithiothreitol (DTT)	Sigma
maleimide derivative of biotin	Sigma
eosin	Molecular Probes
7-diethylamino-4-methyl-3-(4'-maleimidlphenyl)-coumarin	Molecular Probes
monobromo bimane	Molecular Probes

Solvents

Analytical or HPLC grade solvents are commonly used. These are dichloromethane, ethyl acetate, pyridine, N,N-dimethylformamide(DMF), tetrahydrofuran (THF), methanol, hexane, and acetonitrile, which can be obtained from any reputable supplier.

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Site-specific attachment of labels to the DNA backbone

NANCY E. CONWAY, JACQUELINE A. FIDANZA, MARYANNE J. O'DONNELL, NICOLE D. NAREKIAN, HIROAKI OZAKI, and LARRY W. McLAUGHLIN

1. Introduction

Sequence-specific attachment of reporter groups, drug derivatives, or chemically reactive species to DNA sequences has the potential to provide new materials for detailed spectroscopic and biophysical studies as well as a host of new DNA therapeutics and diagnostics. The covalent binding of a variety of such agents at specific locations within the nucleic acid sequence can be achieved by a number of procedures depending on whether the nucleobase, carbohydrate, or phosphate residue is employed as the site of attachment. These procedures often exploit the availability of specific functional groups (such as terminal phosphomonoesters, see Chapter 8) or the reactivity of selected sites on the purine or pyrimidine building blocks (such as the C5 position of pyrimidines, see Chapter 11) in order to attach an appropriate linker or the active agent directly. While the manner in which the nucleic acid is labelled may be dictated by the specific study involved, in general the principles of simplicity and versatility are best employed to guide the choice of labelling procedure.

Some consideration should also be given to the structural effects the label or agent will have the nucleic acid. For example, the addition of an agent or label to a terminal phosphomonoester would be unlikely to alter the structure or stability of a double-stranded or even triple-stranded complex. On the other hand, internal labelling may be more advantageous if the product complex involves an interstrand covalent cross-link or an intercalating agent. Labelling of a site within a sequence has generally relied upon the synthesis of a modified nucleoside building block in which a linker is incorporated for attachment of the label. However, in some cases this can destabilize the duplex structure, for example when the exocyclic amino group of the cytosine residue was used as a site of attachment, the labelled helices exhibited biphasic melting curves (1, 2) suggesting local or even global structural

modulation. Therefore, the nature of the target nucleic acid species to be labelled, or the complex to be formed in the presence of the labelled DNA or RNA sequence, such as single- or double-stranded DNA or RNA, antisense complexes, hairpin structures or triple helix complexes should also influence the method chosen for labelling.

In the present chapter we will describe a series of experiments which allow for the labelling of the phosphate backbone of DNA sequences at single or multiple sites in a sequence-specific manner. These procedures provide versatile labelling at virtually any position within a sequence and are simple enough to be incorporated into standard solid phase based chemical synthesis procedures designed for nucleic acids.

1.1 Labelling techniques directed towards the DNA backbone

The attachment of labels or other agents to the DNA backbone offers a number of advantages over the modification of a terminal phosphomonoester or the modification of a base residue of a nucleoside building block. By using internal phosphodiesters instead of terminal phosphomonoesters to attach the label or agent, virtually any site within the sequence is amenable for introduction of the desired functionality. The phosphate residues are not involved in inter-strand base pairing so the attachment of a linker or label at such sites should not drastically alter the stability of the nucleic acid complex. Sites for backbone labelling can be incorporated into the nucleic acid sequence during the assembly of the sequence by standard chemical techniques and is not limited by the time consuming process involving the synthesis of a modified nucleoside or nucleoside analogue containing the desired linker or label. Multiple labels can also be incorporated without increasing the complexity of the procedure.

In addition, modification of the prochiral phosphodiester residue with a single moiety creates a chiral site of two phosphorus diasteroisomers, (R_p and S_p). Although for many experiments, stereochemically pure derivatives are not essential to obtain the desired information, in cases where stereochemistry is important, the preparation and isolation of each diastereoisomer can be achieved as described in this chapter. The ability to use a singly labelled phosphotriester diastereoisomer can also have certain advantages. For example, with double-stranded structures one phosphorus stereoisomer would direct the covalently attached derivative towards the major groove of an A or B form helix while the second diastereoisomer would direct the agent towards the minor groove. If the desired agent binds or reacts preferentially in one of the helix grooves, stereochemical labelling of the backbone can assist in enhancing the desired reactivity.

Presently two approaches have been employed to introduce labels into the backbone of DNA sequences. Letsinger (3) has described the oxidation of an

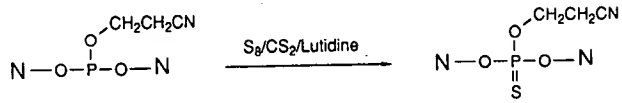
H-phosphonate to the N-substituted phosphoramidate with the desired agent tethered via the nitrogen and others have used his approach (4). Reaction with the substituted amine occurs, instead of other forms of oxidation, immediately after the introduction of the H-phosphonate linkage. The phosphoramidate is stable to the chemical DNA synthesis and deprotection conditions and yields the desired modified sequence. Our own recent work has involved the use of phosphorothioates (5–9) as sites for alkylation by the label of interest and this approach has also been used by others (10). In this case a phosphoramidite coupling is followed by oxidation with sulphur (13–15) to generate the phosphorothioate diester [H-phosphonate chemistry followed by sulphur oxidation (11,12) can also be employed if phosphothioate diesters are introduced at each site in the oligodeoxynucleotide]. After completion of the synthesis, deprotection, and isolation of the fragment, the phosphorothioate is amenable to alkylation (at sulphur) by a variety of functional groups. Both of these procedures result in DNA fragments carrying the agent of interest covalently bound to a specific internucleotide phosphate residue.

In principle either the H-phosphonate or the phosphoramidite procedure can be used to incorporate multiple labels into the nucleic acid fragment. By using DNA fragments containing multiple phosphorothioate diesters we have described a technique (6) which allows the incorporation of hundreds or more bimane fluorophores (one at each phosphorus residue) which results in highly sensitive fluorometric detection of nucleic acids in polyacrylamide gels.

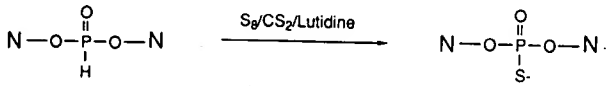
The remainder of this chapter will describe the procedures necessary to modify the DNA backbone at single or multiple sites using one of the methods described above.

2. The synthesis of oligodeoxynucleotides containing phosphorothioate diesters

The synthesis of an oligodeoxynucleotide containing phosphorothioate diesters can be accomplished by both chemical (13–15) (see Chapter 4) and enzymatic (16) means. The methodology in this section employs the chemical synthesis of small oligomers with one or several specifically placed internucleotidic phosphorothioate diesters. Phosphorothioate triesters can be introduced by oxidizing the intermediate phosphite triester obtained during standard phosphoramidite chemistry with elemental sulphur (13–14) to generate a pentavalent phosphorus in which the phosphorothioate triester exists as a thione:



Recent work (15, 17) has also indicated that oxidation to the phosphorothioate triester proceeds rapidly with 3H-1,2-benzodithiol-3-one 1,1-dioxide which exhibits greater solubility than elemental sulphur in organic solvents. However, at present this derivative is not readily available, so the procedures described here employ elemental sulphur. The thione formed in this manner is stable to the subsequent oxidation steps necessary to generate internucleotidic phosphodiesters (as judged from ^{31}P-NMR experiments) in the remainder of the oligodeoxynucleotide. Phosphorothioates can also be formed from the reaction of an internucleotidic H-phosphonate with elemental sulphur (11,12):



In this approach the H-phosphonate is oxidized to the phosphorothioate diester. This species is not compatible with subsequent oxidations employing H_2O/I_2/THF/lutidine. However, it is perfectly adequate for the synthesis of oligonucleotides with a uniform phosphorothioate backbone as described in Chapter 4.

2.1 Synthesis of an oligodeoxynucleotide containing a single internucleotidic phosphorothioate diester

The sequence of interest is prepared by automated DNA synthesis using standard phosphoramidite chemistry on controlled pore glass with chain elongation occurring in the 3′ to 5′ direction as described in Chapter 1 in this book. The program is interrupted at the appropriate step in order to introduce the phosphorothioate by oxidation with sulphur (see also Chapter 4). The solution containing the sulphur oxidant is added manually to the column. This is done because the sulphur will tend to precipitate out of the carbon disulphide/lutidine solution and when this occurs in the delivery lines of a DNA synthesis machine the lines become plugged. As noted above, the 3H-1,2-benzodithiol-3-one 1,1-dioxide derivative described by Iyer *et al.* (15,17) exhibits better solubility in organic solvents and can be used directly in the automated synthesis. The following procedure uses the simpler sulphur oxidant.

Protocol 1. Oxidation of the intermediate phosphite triester to the phosphorothioate triester

Materials

- Four 1 ml glass syringes
- Elemental sulphur, carbon disulphide, lutidine

Protocol 1. *Continued*

- Additional reagents necessary for automated phosphoramidite DNA synthesis.

Method

1. Assemble the sequence in the normal manner until the nucleoside residue 3′ to the desired phosphorothioate linkage has been added, capped and oxidized to the phosphate triester. Couple the nucleoside residue 5′ to the phosphorothioate and follow with a capping step. At this point the synthesis is interrupted and the column removed from the machine. With many DNA synthesizers the machine can be programmed to interrupt at this point in the cycle.
2. Fill a 1 ml syringe with a solution of 2.5 M elemental sulphur in carbon disulphide/lutidine (1:1) prepared shortly before use (to prevent extensive precipitation of the sulphur) in an oven dried flask (for a 1 μmol synthesis) according to the following:
   - 0.401 g elemental sulphur (Aldrich gold label: 99.999% pure).
   - 2.5 ml carbon disulphide.
   - 2.5 ml lutidine (previously distilled over Ninhydrin followed by distillation over KOH).

   Insert a second empty 1 ml syringe into the outlet of the synthesis column and insert the filled syringe into the inlet. Add the sulphur solution to the synthesis column such that any excess solution is collected in the second syringe. Allow the oxidation reaction to continue for 30–60 min. Every 5–10 min pass the solution back and forth through the column.
3. After 30–60 min wash the synthesis column with carbon disulphide/lutidine (1:1) using two syringes as described above. This washes the excess sulphur from the column and redisolves any precipitated sulphur.
4. Remove the excess solvent from the column prior to its attachment to the machine. Attach the column to the machine and wash the column manually with acetonitrile for 120 s and then re-initiate the synthesis program (excluding the first oxidation step).
5. After assembly of the oligodeoxynucleotide, deprotect, and purify the fragment as described for unmodified oligodeoxynucleotides. Use the isolation procedure in which the 5′-terminal DMT group is not removed from the sequence.

In most cases there is virtually no difference in retention time on a reversed phase column for the modified (phosphorothioate containing) or the unmodified DMT containing oligodeoxynucleotide. With increasing numbers

of phosphorothioate diesters some peak broadening is observed presumably as a result of the presence of numerous diasteroisomers.

After isolation and desalting, the purity of the phosphorothioate containing oligomer can be analyzed by reversed phase chromatography using Buffer A: 0.02 M KH_2PO_4 pH 5.5, Buffer B: 0.02 M KH_2PO_4, pH 5.5 containing 70% methanol with a flow of 1.5 ml/min and a gradient of 0–100% Buffer B in 60 min as previously described. The oligomer containing a single pair of phosphorothioate diasteroisomers will elute as two peaks with only slight separation between isomers or as a broad peak with little or no separation depending upon the length of the fragment.

2.2 Synthesis of stereochemically pure oligodeoxynucleotides containing phosphorothioate diesters

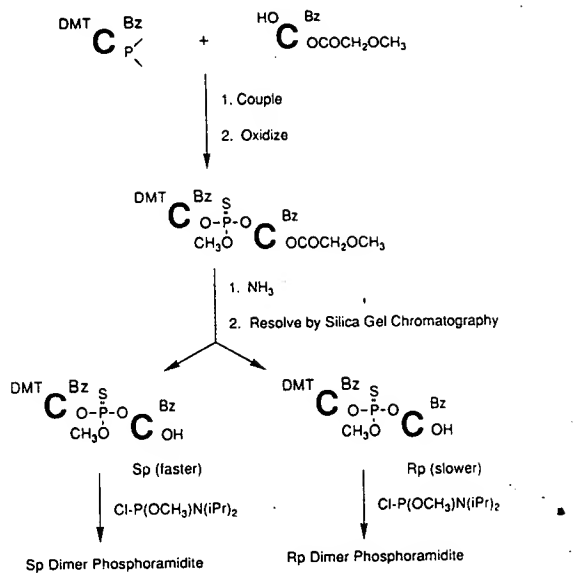
The chemical oxidation of the intermediate phosphite triester with sulphur results in a roughly equal mixture of the R_p and S_p diastereoisomers. Reaction of this mixture with the desired label or agent will yield a corresponding isomeric mixture of labelled DNA fragments (6). With short fragments it is sometimes possible resolve the two diastereoisomers chromatographically but this is a less than ideal approach, and with longer fragments there is often no detectable difference in retention between the labelled R_p and labelled S_p isomer. In order to effectively generate stereochemically pure DNA labelled at the phosphorothioate, it is most efficient first to generate the isomerically pure phosphorothioate dinucleotide building block with the techniques first reported from the laboratory of Fritz Eckstein (18,19). In this procedure the desired nucleoside dimer containing a phosphorothioate diester is prepared in solution followed by separation of the two diastereoisomers. After conversion of each diastereoisomer into the appropriate phosphoramidite, the dimer of known chirality can be incorporated into the DNA fragment (see *Scheme*).

Protocol 2. Preparation and purification of the fully protected R_p and S_p isomers of the dimer Cp(s)C, and its conversion to the corresponding phosphoramidite derivative

Materials

- Methoxyacetic acid, DCC, anhydrous pyridine
- 4,4′-Dimethoxytrityl-N-benzoyl-2′-deoxycytidine
- Elemental sulphur, carbon disulphide, pyridine
- Acetic acid, concentrated aqueous ammonia, dioxane
- Silica gel 60 (0.40–0.63 μM or finer than 0.63 μM)
- Methyl-N,N-diisopropylchlorophosphoramidite
- Dichloromethane, methanol, hexane

Scheme: Preparation of Pure Sp or Rp Phosphorothioate Diesters



Protocol 2. *Continued*

A. *Synthesis of methoxyacetic anhydride*

Combine 1.217 ml (15.86 mmol) of methoxyacetic acid and 6 ml of dichloromethane. Add 1.6336 g (7.93 mmol) of DCC over a 10 min period. Stir the reaction for 1 h under anhydrous conditions. Filter the solution to remove the urea derivative.

B. *Synthesis of N-benzoyl-3′-O-(methoxyacetyl)-2′-deoxycytidine*

1. Dissolve 2.0080 g (3.172 mmol) of 4,4′-dimethoxytrityl-N-benzoyl-2′-deoxycytidine in anhydrous pyridine. Add the methoxyacetic anhydride (see above) to the pyridine solution under anhydrous conditions. Monitor the reaction by TLC (9:1 CH_2Cl_2:CH_3OH). If the reaction is not complete after approximately 4 h, add an additional 7.5 mmol of methoxyacetic anhydride and stir for an additional 2 h. Evaporate the reaction mixture to dryness under high vacuum and co-evaporate twice from toluene.

Protocol 2. *Continued*

2. Add 20 ml of 80% acetic acid to the residue and stir for 2.5 h. When the reaction is complete (TLC), add 4 ml of H_2O to quench the reaction and evaporate the mixture to dryness. Dissolve this residue in 50 ml of dichloromethane and wash it twice with 5% aqueous sodium bicarbonate and dry. Evaporate the organic phase to dryness and purify the residue on a 30 g column of silica gel. Elute the product using 100 ml portions of dichloromethane using an increasing methanol gradient.

C. *Synthesis of R_p and S_p isomers of the fully protected d[Cp(s)C] dimer*

1. Dry 0.4836 g (1.2 mmol) of N-benzoyl-3′-O-(methoxyacetyl)-2′-deoxycytidine, 1.4312 g (1.8 mmol) of 5′-O-(4,4′-dimethoxytrityl)-N-benzoyl-3′-O-(methoxy phosphoramidite)-2′-deoxycytidine, and 0.3362 g (4.8 mmol) of tetrazole (Aldrich, gold label) in vacuum oven at 50 °C for 12 h. After this time, add 20 ml of anhydrous acetonitrile to dissolve all reagents. Monitor the reaction with TLC. The reaction is generally complete within minutes but is often stirred for an hour or more.
2. Dissolve 0.3847 g of elemental sulphur (Aldrich, gold label) in 16 ml of anhydrous carbon disulphide/pyridine solution (1:1). Add this solution dropwise to the reaction mixture, (some sulphur precipitation results). Stir this reaction for 2 h and then filter the solution to remove the precipitate. Evaporate the yellow solution to dryness under high vacuum and co-evaporate twice from toluene. Dissolve the residue in 50 ml of dichloromethane and wash twice with 5% aqueous sodium bicarbonate, twice saturated sodium chloride, dry the organic phase (sodium sulphate) and evaporate it to dryness.
3. Dissolve the residue in 20 ml of dioxane and add 5 ml of 25% aqueous ammonia. Allow this to stir for approximately 2.5 h while monitoring by TLC. When the reaction is complete, evaporate the solution to dryness.
4. Purify the product by chromatography on a column of silica gel 60.

D. *Synthesis of the methyl phosphoramidite derivative of the fully protected d[Cp(s)C] dimer*

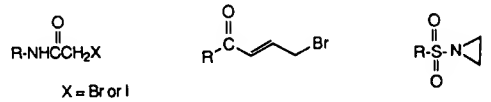
1. To 200 mg (200 mmol) of a dimer in 5 ml dry dichloromethane containing 150 µl of diisopropylethylamine add 75 µl (525 µmol) of N,N-diisopropyl-methylphosphonamidic chloride. Stir the reaction mixture for 2 h at ambient temperature, TLC analysis should indicate that the reaction is largely complete. Stop the reaction with 1 ml of methanol, evaporate to dryness, and chromatography the residue on a column of silica gel 60 using dichloromethane (containing 1% triethylamine) and a methanol gradient.
2. Pool the fractions containing product and evaporate to dryness. Dissolve

Protocol 2. *Continued*

the residue in a minimum amount of dichloromethane and precipitate into hexane. Filter the precipitate, dry it under vacuum, and store it in a desiccator (over KOH). Yields were typically between 50 and 70%.

3. Reactions of phosphorothioate diesters

Oligodeoxynucleotides containing a uniquely placed covalently bound reporter group tethered to the DNA backbone by alkylation of a phosphorothioate diester are easily obtained by incubation of a phosphorothioate-containing oligodeoxynucleotide with the probe of choice in aqueous or largely aqueous solution within a pH range of 5–8 and from 25 to 50 °C. Our preliminary experiments employed the simplest phosphorothioate diester Tp(s)T in order to establish the rates of reactivity with different functional groups under a variety of conditions. Working with the dimer is advantageous because alkylation of the sulphur residue can be easily monitored by HPLC with the large shift in retention time of the labelled versus unlabelled phosphorothioate diester. However, the protocols described in this section are designed for sequences from 10 to 24 residues. Three functional groups can be employed to covalently introduce a label or other agent to the DNA backbone by alkylation of the phosphorothioate diester. Reagents containing γ-bromo-α,β-unsaturated carbonyls, iodo or bromo acetamides, or aziridinylsulphonamides, function effectively to alkyate the sulphur residue and produce the corresponding phosphorothioate triester:



Both the R_p and S_p diastereoisomers exhibit equal reactivity in all reactions monitored to date.

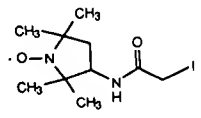
The concentration and amount of label required for rapid and efficient reaction with the phosphorothioate diester varies significantly depending upon the functional group employed for the alkylation since the rates of competing hydrolytic reactions also vary. Additionally the aqueous character of the reaction mixture will depend largely upon the solubility of the label as well as the oligodeoxynucleotide. Aqueous or dimethylformamide solutions can commonly be employed to dissolve both the oligodeoxynucleotide and the label or agent of interest. The amount of DMF required in a given reactions is dependent upon the solubility of the appropriate reagent. However, with DMF concentrations greater than 55%, precipitation of the oligodeoxynucleotide may become problematic. Three protocols are described

in this section; the first employs a largely water soluble spin label to modify a 12mer, the second a hydrophobic fluorophore to modify a 24mer and the third uses a drug derivative to alkylate a 12mer.

Protocol 3. Attachment of the PROXYL spin label to a phosphorothioate containing 12mer

Materials

- 3-(2-Iodoacetamidomethyl)-PROXYL
- 100 mM potassium phosphate, pH 6.0
- d[CGCA(s)AAAAAGCG]



Method

1. Prepare the reaction mixture in a 0.5 ml Eppendorf tube as described below:

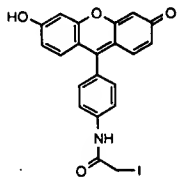
Reagent (mM)	Volume (µl)	Amount (µM)	Equivalents	Final concentration (mM)
PROXYL (25) in 30% aqueous DMF	100	2.5	31	12 14% DMF
12mer (1.35)	60	0.08	1	0.39
Buffer (100)	50			24
Total:	210 µl			

2. Seal the Eppendorf tube with Parafilm and completely submerge it in a water bath at 50 °C for 6 h.
3. At this point the reaction is 80–90% complete and product is isolated by HPLC as described in *Protocol 6*.

Protocol 4. Attachment of 5-iodoacetamidofluorescein to a phosphorothioate containing 24mer

Materials

- 5-iodoacetamidofluorescein (5-IAF)
- 100 mM Tris–HCl, pH 8.0
- d[CGAACT(s)AGTTAACTAGTACGCAAG]



Protocol 4. *Continued*

Method

1. Prepare the reaction mixture in a 0.5 ml Eppendorf tube as describe below:

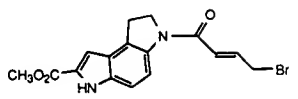
Reagent (mM)	Volume) (µl)	Amount (µM)	Equivalents	Final concentration (mM)
5-IAF (50) in DMF	30	1.5	16.7	15 30%
24mer (2.0)	45	0.09	1	0.9
Buffer (100)	25			25
Total:	100 µl			

2. Seal the Eppendorf tube the Parafilm, cover with aluminum foil and completely submerge it in a water bath at 50 °C for 24 h.
3. At this point the reaction is 80–90% complete and product is isolated by HPLC as described in *Protocol 6*.

Protocol 5. Attachment of a CC-1065 analogue to a phosphorothioate containing 12mer

Materials

- CDPI-Br
- 100 mM sodium acetate, pH 5.0
- d[CGCA(s)AAAAAGCG]



Method

1. Prepare the reaction mixture in a 1.0 ml Eppendoft tube as described below:

Reagent (mM)	Volume) (µl)	Amount (µM)	Equivalents	Final concentration (mM)
CDPI-Br (20) in DMF	156	3.1	20	3.7
12mer (0.3)	525	0.16	1	0.19
Buffer (100)	43			10
DMF	100			30% total
H_2O	26			
Total:	850 µl			

2. Seal the Eppendorf tube with Parafilm and completely submerge it in a water bath at 50 °C for 12 h.
3. At this point the reaction is 80–90% complete and product is isolated by HPLC as described in *Protocol 6*.

3.1 Analysis and purification of the labelled oligodeoxynucleotides

The reactions are typically monitored and the products isolated by HPLC using reversed-phase chromatography. This method is advantageous since alkylation of a phosphorothioate diester converts the charged diester to the neutral triester. Often the alkylating moiety is itself hydrophobic such that the product oligodeoxynucleotide has more hydrophobic character than the unlabelled starting material and consequently elutes from the column with an increased retention time. For example, resolution of the labelled and unlabelled dodecamers obtained after reaction of the single-stranded 12mer with 3-(2-iodoacetamido)-PROXYL is illustrated in *Figure 1*. This analysis additionally illustrates a common observation with diastereomeric mixtures, in that the R_p and S_p phosphorothioate diesters are not resolved by the column, but addition of a label at the chiral centre enhances separation such that resolution of the two labelled diastereoisomers occurs. In some cases, as

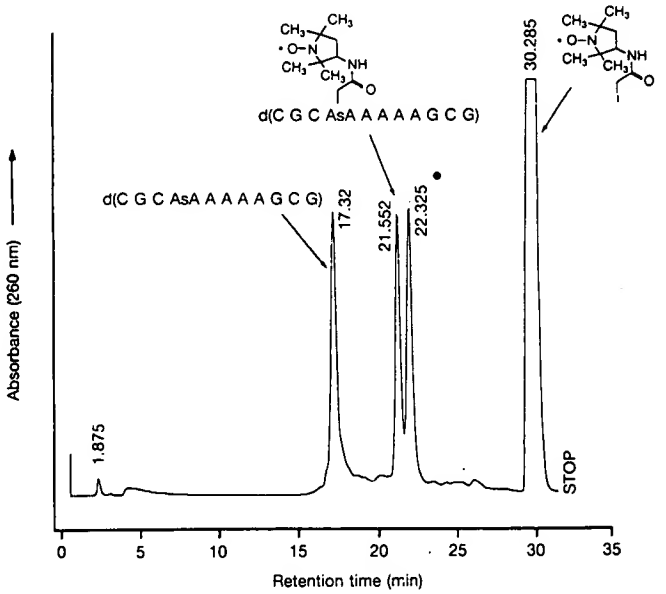


Figure 1. Analysis of the reaction mixture of a dodecamer containing a single phosphorothioate diester and the iodoacetamidoPROXYL spin label after two hours of reaction. Column conditions similar to that described in *Protocol 6*.

illustrated by the chromatogram of *Figure 1*, it should be possible to purify the two isomeric compounds by HPLC. However, this is unlikely to be an effective general method. Typically such isomer resolution decreases with increasing chain length (see also the analysis of the labelled 24mer, *Figure 3*). Isolation of chirally pure material is much more efficient if the chiral phosphorothioate dinucleotide building block is prepared for incorporation into the sequence as previously reported (18, 19) and described above in *Protocol 2*.

Effective resolution of the labelled and unlabelled material can often be achieved with a column equilibrated in 20 mM phosphate and employing a methanol gradient as described below:

Protocol 6. HPLC analysis and isolation of the labelled oligodeoxynucleotide derivatives

Materials

- 4.6 × 250 mm reversed phase column (ODS-Hypersil)
- 1.0 M potassium phosphate pH 5.5
- HPLC grade methanol
- Gradient HPLC system (Beckman)
- Membrane filtration set-up (Millipore)
- Sep-Pak or Nensorb cartridge

Method

1. Prepare two buffers by appropriate dilution of the stock solution and addition of methanol: Buffer A: 20 mM potassium phosphate pH 5.5. Buffer B: 20 mM potassium phosphate pH 5.5, 70% methanol.
2. Filter both buffers through a 0.45 μm membrane filter to remove particulates.
3. Wash the column with buffer B and then re-equilibrate with buffer A.
4. Programe the HPLC system to elute the column under the following conditions: Flow rate: 1.5 ml/min, Linear gradient: 0–100% buffer B in 60 min.
5. For isolation of the labelled fragment, collect the appropriate peak, evaporate it to dryness. Prepare a Sep-Pak (or Nensorb) column in the following manner: Attach a 10 ml plastic syringe cylinder to the Sep-Pak column as a solvent reservoir. Wash the column with 20 ml of methanol followed by 20 ml of double-distilled water. Dissolve the residue containing the labelled fragment in distilled water and add this solution of the Sep-Pak column.

Protocol 6. *Continued*

6. Wash the Sep-Pak (Nensorb) column with 10 ml of water to remove the phosphate buffer followed by 10 ml of 50% aqueous methanol (higher concentrations may be required in some instances depending upon the moiety bound) to elute the labelled DNA fragment.
7. Lyophilize to dryness and store at −20 °C.

HPLC resolution of the labelled and the unlabelled fragments has been very successful but ultimately may be limited by the length of the fragments and the hydrophobic character of the attached derivative. A 24mer labelled with a iodoacetamidofluorescein derivative can be easily resolved from the unlabelled 24mer (*Figure 2*). However, the iodoacetamidodansyl derivative of a dodecamer was more difficult to obtain in purified form. In the latter case, modification of the 12mer with iodoacetamidodansyl converts the negatively charged phosphorothioate diester to the neutral triester which should allow its separation from unlabelled 12mer. However, the dansyl group itself carries a negatively charged sulphonic acid residue which appears to obscure differences in polarity between the labelled and unlabelled

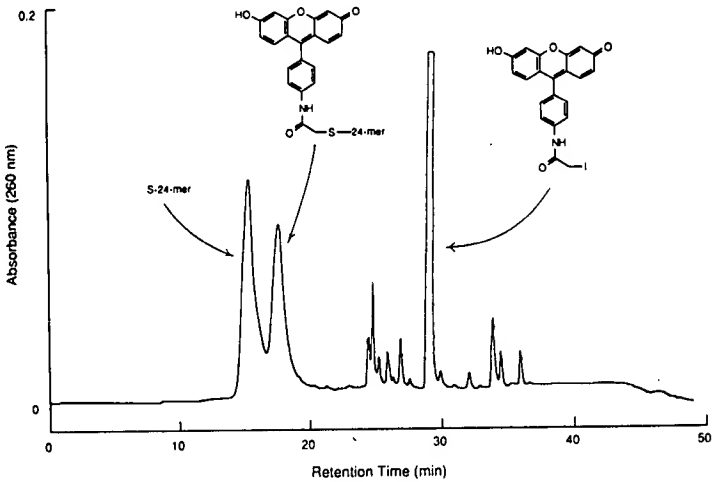


Figure 2. Analysis of the reaction mixture of a 24mer containing a single phosphorothioate diester and the iodoacetamidofluorescein fluorophore after six hours of reaction. Column conditions were similar to that described in *Protocol 6* (small unlabelled peaks are present in the fluorophore preparation or are related hydrolysis products).

fragments. By using a shallower methanol gradient than the described in *Protocol 6* (0–30% methanol in 60 min), the dansyl labelled dodecamer could be purified (*Figure 3*), however similar success with this label and longer fragments may be more difficult to achieve.

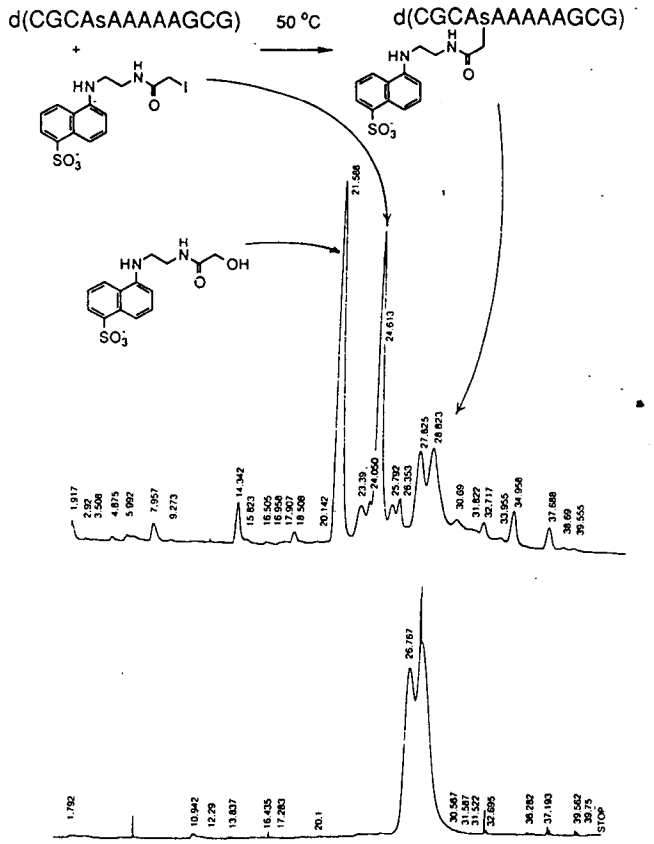


Figure 3. Above: analysis of the reaction mixture of a dodecamer containing a single phosphorothioate diester and the bromoacetamidodansyl fluorophore after four hours of reaction. Column conditions similar to that described in *Protocol 6* but incorporated a 0–35% methanol gradient in 60 min (small unlabelled peaks were present in the fluorophore preparation). Below: HPLC analysis of the isolated labelled product.

3.2 Variations in reactivity

Alkylation of the phosphorothioate diester is generally more efficient in a simple dimer or a single-stranded oligodeoxynucleotide, than for a self complementary or double-stranded (for example an eicosomer) fragment. This difference in reactivity can be dramatic at ambient temperature where a self-complementary eicosomer (20mer), or longer fragment, is largely double-stranded and suggests that nucleic acid secondary structure can reduce the accessibility of the internucleotidic phosphorus residue to the reporter group. This difficulty can be overcome by heating the reaction mixture to 50 °C and presumably disrupting the DNA helical structure without any noticeable side reactions. Since reaction conditions which incorporate elevated temperatures enhance reactivity as well as break down secondary structure, we routinely use incubation temperatures of 50 °C.

A large portion of the phosphorothioate diester is converted to the labelled phosphorothioate triester within the first few hours of reaction as illustrated for the 24mer in *Figure 4*. However, reaction mixtures are typically and conveniently incubated overnight. Reactions yields under such conditions are typically 80–90% as observed in the plot of *Figure 4* for a 24mer.

Under these conditions we cannot detect any side reactions when the haloacetamido or γ-bromo-α,β-unsaturated carbonyl derivatives are utilized for labelling. However, the HPLC analyses of reactions employing the aziridinyl sulphonamides indicates the presence of minor products in addition to the major product, the phosphorothioate triester. This is not surprising since aziridinyl derivatives are known to modify DNA bases. Using HPLC it

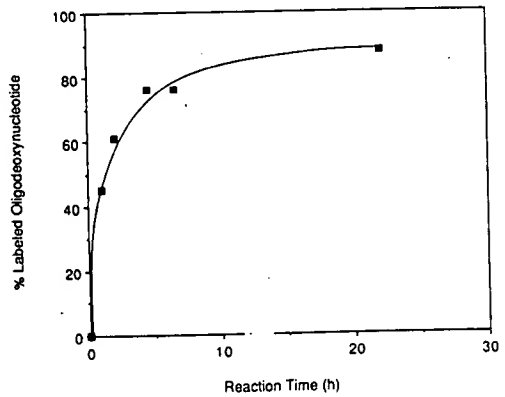


Figure 4. Graph of the extent of product formation for the fluorescein labelled 24mer after various reaction times (for conditions see *Protocol 5*).

is still possible to isolate fragments labelled with aziridinyl sulphonamides in high purity, however, due to the additional reactivity exhibited by these compounds we prefer to use haloacetamide or γ-bromo-α,β-unsaturated carbonyl derivatives to attach the label of interest to the DNA backbone.

3.3 Stability of the product phosphorothioate triesters

In order for the oligodeoxynucleotides labelled by these procedures to the valuable for further studies, including those of structure and dynamics, it is required that the phosphorothioate triesters exhibit reasonable stability in aqueous solutions. Hydrolysis of the phosphorothioate triester proceeds almost exclusively by P-S bond cleavage to yield the native oligodeoxynucleotide with an internucleotidic phosphodiester in place of the phosphorothioate triester and the corresponding sulphydryl derivative of the reporter group [minor amounts of chain cleavage also occur and this phenomenon has been the basis for phosphorothioate based DNA sequencing (20)]. We have not quantified the fate of the reporter group in these experiments but have monitored the conversion of phosphorothioate triester to phosphate diester.

Previous experiments had indicated that the triester formed from the dimer, d[Tp(s)T], was stable for long periods of time at pH values below 7 but was rapidly hydrolysed ($t_{1/2} < 30$ min) at pH 11. To compare the stability of a simple dimer with that of a dodecamer and self complementary eicosomer (20mer), the appropriate derivatives are incubated at pH values of 7.0, 8.0, and 10.0, at ambient temperature over a 24 h time period. Under these conditions the stability of the phosphorothioate triester increases in the order: dimer < dodecamer < self complementary (*Table 1*). The stability of the modified DNA will vary with the character of the label. The hydrolysis results reported in *Table 1* employed the iodoacetamido-dansyl group which is one of the least stable labels we have examined. In general, the triester resulting from reaction with the γ-bromo-α,β-unsaturated carbonyl exhibited stability similar to that of the haloacetamido labelled derivatives, while that resulting from reaction with the aziridinyl sulphonamide is significantly more stable.

Table 1. Stability of phosphorothioate labelled DNA

	Quantity of labelled oligodeoxynucleotide remaining after incubation at ambient temperature for 24 h		
pH	**Tp(s)T-Dansyl**	**12mer-danysl[a]**	**20mer-dansyl[b]**
7.0	90%	99%	> 99%
8.5	71%	69%	98%
10.0	0%	0%	37%

[a] Single-stranded
[b] Double-stranded

3.4 Thermal stability of labelled phosphorothioate triester-containing DNA

When a probe is introduced into a native biopolymer it is necessary to consider the structural implications and possible perturbation of the structure which might occur. Attachment of a label to the outer surface of the biomolecule, in this case to the phosphorus residue, may be of minimal structural consequence, but a large group may still interfere with proper Watson–Crick base pairing. As an example of the stability of backbone labelled oligodeoxynucleotide helices, a number of modified sequences have been investigated to determine if a backbone modification generally destabilizes the duplex structure. The thermal melting curves (helix-to-coil transitions) obtained for a dodecamer containing a single PROXYL spin label, a drug derivative or a self complementary eicosomer containing two labelled phosphorothioate triesters, indicate that incorporation of a reporter group does not generally impart any significant instability to the helical structure. Thermal melting points are measured in 1.0 M NaCl and 10 mM KH_2PO_4, pH 7.0. The unlabelled dodecamer exhibits a T_m (55 °C) which is indistinguishable from the T_m values obtained for the PROXYL labelled or drug labelled dodecamer helices (T_ms of 55 and 54 °C respectively) under the same conditions. The T_m value for the self complementary eicosomer with two labels shows a slight increase of 1.5 °C in comparison to the unlabelled fragment. The conversion of one or two negatively charged diesters to the neutral triesters within 10–20 base pairs does not appear to have any significant structural implications.

4. Applications for site-specifically modified DNA fragments

Site-specifically labelled DNA fragments can be valuable in a number of different biophysical studies including the study of nucleic acid structure and dynamics as well as protein–DNA or ligand–DNA complexes. The case of attachment of reporter groups such a fluorophores should simplify the incorporation of donor and acceptor groups for use in energy transfer studies on a variety of unusual structures and complexes. Additionally, the sequence-specific attachment of reactive agents such as bifunctional alkylating agents, incorporation of donor and acceptor groups for use in energy transfer studies series of modified antisense oligodeoxynucleotides as an approach to antiviral therapy. Furthermore, such modified DNA fragments may soon initiate a new class of potentially sensitive materials for use as what might be termed DNA diagnostics or therapeutics. In the present chapter we will describe the use of site-specifically labelled DNA to monitor the sequence-specific binding by a DNA repressor protein.

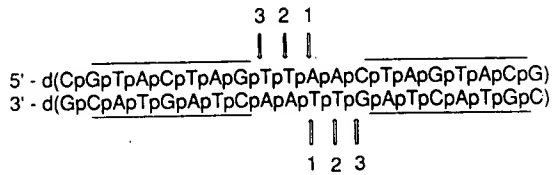
4.1 Detection of sequence-specific protein–DNA binding

One of the best understood mechanisms of genetic expression is that described by the operon model in which the expression of a particular enzyme or series of enzymes is controlled by the binding of a repressor protein to an operator sequence which in part overlaps the RNA polymerase promoter sequence. With the repressor protein bound, RNA polymerase binding is inhibited and no expression of the gene occurs. In the absence of the repressor protein, RNA polymerase can bind and the gene is expressed.

4.2 Detection of binding between the tryptophan repressor and operator

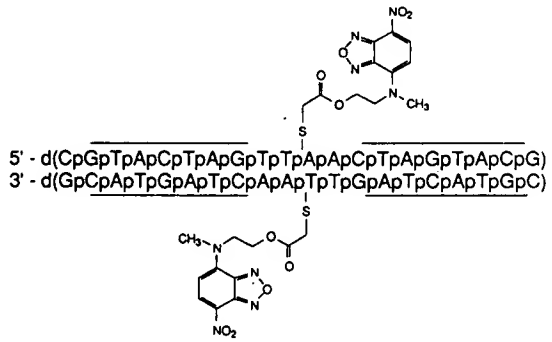
The tryptophan operon controls expression of the bacterial enzymes responsible for the synthesis of the amino acid tryptophan. Binding of the tryptophan repressor protein to the operator sequence is modulated by the tryptophan amino acid which functions as a co-repressor. Upon binding tryptophan the repressor protein undergoes a conformational change which dramatically enhances its affinity for the operator sequence. Unfortunately there are not presently many efficient assays for measuring solution binding between macromolecules such as proteins and nucleic acids. Most assays such as nitrocellulose filter binding or gel shift assays involve trapping the protein–nucleic acid complex. It would be desirable if the protein–nucleic acid complex could be detected directly in solution, ideally under equilibrium conditions. In order to assess such possibilities we have prepared a series of site-specifically labelled operator sequences. In this case the choice of site to label was dictated by the crystal structure of the tryptophan repressor–operator complex. We prepared three site-specifically labelled operator sequences by placing a phosphorothioate diester between residues −1 and +1 (site 1 below), between residues −1 and −2 (site 2 below) and between residues −2 and −3 (site 3 below):

Trp Operator



These sites were chosen since the crystal structure indicated that the repressor protein did not directly contact this area of the operator and we did not want the fluorophore to inhibit binding by the protein (the lines above mark the

bases of the operator directly contacted by the repressor). However, it was anticipated that the fluorophore would be very close to the protein upon binding such that complex formation might alter the environment of the fluorophore. With a fluorophore sensitive to such change in the local environment, it was possible that protein binding would be reported by changes in the characteristics of the emitted fluorescence. The fluorophore chosen for this study was the iodoacetamido derivative of nitrobenzdiimidazole. Three sequences were prepared with the label linked to the internucleotidic phosphorothioate diester as typified by the example below:



The labelled sequences were prepared by the procedure described in *Protocols 3*, *4*, and *5* of this chapter. After isolation and purification of the labelled fragments as described in *Protocol 6* each of the fragments was titrated with repressor protein under the conditions described below. To the operator solution (0.4 ml containing 10 mM Tris–HCl pH 7.4, 250 mM sodium chloride, 0.5 mM L-tryptophan, 0.1 mg/ml BSA and 5 μM NBD-labelled 20mer) was added 1.6 μl aliquots of the repressor (0.1 ml containing 265 μM tryptophan repressor dimer (in 1[illegible] mM phosphate). After addition and mixing, the fluorescence of the soluti[illegible] was measured. Additions were continued until the concentration of the re[illegible]ssor dimer had reached 10 μM.

As can be observed in *Figure 5*, the fluorescence intensity of the solution increased proportionately with increasing concentrations of repressor protein. When the concentration of the protein equalled that of the operator site the fluorescence increase reached its maximum. At that point, any further increase in repressor concentration did not yield a corresponding increase in fluorescence intensity. During this titration, no change in the excitation or emission maxima for the nitrobenzdiimidazole derivative was observed.

The same titration performed in the absence of the amino acid tryptophan did not produce any measurable change in fluorescence intensity. The

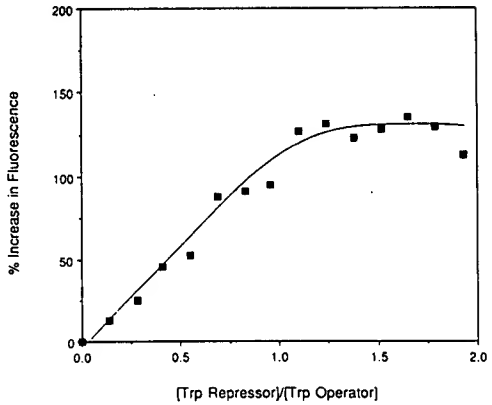


Figure 5. Change in fluorescence of the NBD labelled 20mer with the addition of increasing concentration of the tryptophan repressor.

addition of a non-specific protein (BSA) to a concentration of 125 μM did not result in significant changes in the observed fluorescence. Both of these control experiments argue that the observed increased in fluorescence intensity was directly the result of the formation of the sequence-specific repressor–operator complex.

We additionally compared changes in fluorescence for the titration of sequences, 1, 2, and 3. Both sequences 1 and 2 exhibited similar increases in fluorescence (100–125%) during titration by the repressor. However, sequences 1, 2, and 3. Both sequences 1 and 2 exhibited similar increases in increase in fluorescence). This can be explained in terms of the published repressor–operator complex in that as the fluorophore is moved from position 1 to 2 to 3 it is rotated away from the bulk of the protein present in the complex. Sequences 1 and 2, which place the fluorophore nearer the protein, report a more significant change in environment than does sequence 3. This indicates that the site-specific placement of fluorophores on the DNA backbone can be useful to monitor sequence-specific protein–DNA binding, and may in some cases also be used to map those areas of the DNA helix which are approached most closely by the protein upon binding.

5. DNA containing multiple phosphorothioate diesters

Substitution of one of the non-bridging oxygens of the internucleotidic phosphate diester by a sulphur atom is a relatively conservative modification.

In fact, DNA sequences which contain multiple phosphorothioate diesters are essentially native in structure, and often the physical properties, such as T_m values are virtually indistinguishable from those for unmodified DNA. The substitution of phosphate diesters by phosphorothioate diesters creates a series nucleophilic sites in an otherwise native DNA fragment. However, the presence of multiple phosphorothioates now allows for the introduction of multiple labels, ideally one at each phosphorus residue.

5.1 Synthesis of DNA with multiple phosphorothioate diesters

Phosphorothioates can be introduced into nucleic acids at multiple sites using phosphoramidite or H-phosphonate chemistry by the procedures described in Chapter 4. Multiple phosphorothioate diesters can also be introduced into a DNA fragment enzymatically using polymerases when one or more of the substrate α-thio-2′-deoxynucleotide-5′triphosphates are employed as originally developed in the laboratory of Fritz Eckstein. Replacement of one of the non-bridging oxygens at the α-position of a 2′-deoxynucleotide-5′-triphosphate with sulphur creates two diastereoisomers. All polymerizing enzymes examined to date (including *E.coli* DNA polymerase I or the Klenow fragment, phage T4 and T7 DNA polymerases, *Micrococcus luteus* DNA polymerase, reverse transcriptase, and *Taq* polymerase) use only the S_p diastereoisomer (16, 21, 22) as a substrate (in the presence of a Mg^{2+} cofactor). After polymerization the S_p α-thio-2′-deoxynucleoside 5′-triphosphate, the phosphorothioate diester formed is exclusively in the R_p configuration. In theory the polymerizing enzymes will use only the S_p isomer from a racemic mixture of phosphorothioates, however in practice we have found better results are obtained if pure S_p α-thiotriphosphate is used. This observation is likely a function of the higher purity of α-thiotriphosphate which is obtained if the care is taken to isolate a single stereoisomer rather than any inherent inefficiency exhibited by the enzyme in the presence of the racemic mixture. The procedures described in the section employ the pure S_p α-thiotriphosphates which are readily available (Amersham).

Protocol 7. Enzymatic synthesis of the replicative form of M13mp18 DNA with multiple phosphorothioate diesters in the (−) strand.

Materials

- M13mp18 single stranded DNA
- Universal primer
- Buffer (100 mM Tris–HCl, pH 8.0, 100 mM NaCl, 20 mM $MgCl_2$)
- dNTPαS (dATPαS, dGTPαS, dCTPαS, dTTPαS)

Protocol 7. *Continued*

- ATP
- DNA polymerase I (10 units)
- T4 DNA ligase (6 units)

Method

1. Add DNA and primer to an Eppendorf tube in <10 μl and add 25 μl of buffer. Heat the mixture to 56 °C and then cool it to ambient temperature over a 30 min period. Add all four dNTPαS substrates and ATP such that the reaction volume is 50 μl and the final concentration of each S_p α-thiotriphosphate is 500 μM and ATP is 1 mM. Add DNA polymerase I and T_4 DNA ligase and incubate the reaction mixture at 16 °C overnight (18 h).
2. Heat the reaction mixture to 70 °C and maintain this temperature for 10 min to inactivate the enzymes. Add two volumes of ethanol, incubate at −20 °C for 60 min. Centrifuge at 0 °C for 10 min. Discard supernatant and remove residual ethanol under vacuum. Resuspend the DNA pellet in the appropriate buffer.

In the labelling reaction described in section 6 of this chapter, DNA restriction fragments are labelled at multiple sites with the fluorophore monobromobimane. It has been reported that many restriction endonucleases are sensitive to the presence of a phosphorothioate diester at or near the cleavage site (18, 23, 24). If specific DNA fragments will be prepared from the phosphorothioate containing DNA described in *Protocol 7*, then the protocol must be modified such that only two or three α-thiotriphosphates plus the unmodified triphosphate(s) are used as substrates. This will allow the construction of the RF form of the DNA which still contains many phosphorothioate diesters, but maintains an unmodified phosphate diester(s) at or near the site of restriction endonuclease hydrolysis.

6. Fluorescent labelling of phosphorothioate diesters for the detection of DNA

Fluorophores represent an attractive class of reporter molecules for the detection of nucleic acids because they are stable and directly detectable and could potentially replace radioisotopic labelling procedures in some assays. Many procedures have been described for the introduction of a single fluorophore (25), and in some cases a number of fluorophores (26, 27) into nucleic acids. However, fluorophores do not typically allow the detection of small quantities of nucleic acids ($<10^{-15}$M) in the absence of sophisticated

detection systems; and the high sensitivity of radioisotopes for detection by autoradiography typically allows the observation of femtomolar quantities of nucleic acid (28).

6.1 Introduction of multiple fluorophores into DNA containing phosphorothioates

Highly sensitive detection of DNA should be expected if multiple fluorophores, ideally one at each phosphorus residue, can be introduced. In principle, multiple fluorophores can be introduced into DNA fragments containing multiple phosphorothioate diesters essentially under the conditions described in section 3 of this chapter. However, the isolation of DNA fragments labelled in this manner is problematic. After fluorescent labelling, a DNA fragment of hundreds of nucleoside residues contains hundreds of covalently bound fluorosphores (*Figure 6*). Since the charged phosphorothioate diesters are converted to neutral phosphorothioate triesters upon labelling, such fragments would have severely altered properties including but not limited to solubility. Because of the inherent problems which accompany the purification of DNA containing multiple labels bound to the phosphorus residues, we have examined a procedure by which the fluorophores are introduced *in situ*, only after the assay of interest. In the present chapter a technique for introducing multiple fluorophores into DNA after resolution of DNA fragments by polyacrylamide gel electrophoresis is described.

6.2 Post-assay labelling for high detection sensitivity

A simple protocol has been developed which allows for the covalent introduction of multiple fluorescent markers into DNA fragments after gel

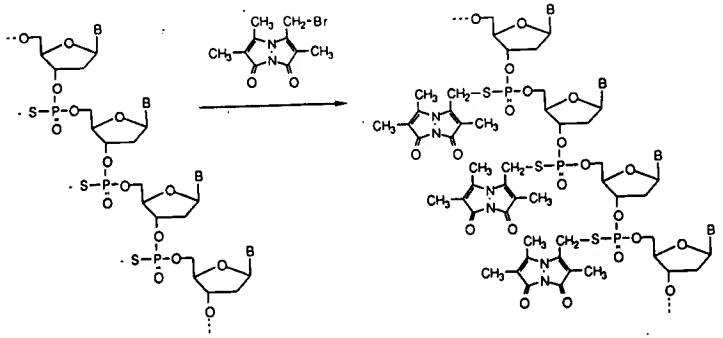


Figure 6. Structure of a portion of single-stranded DNA which has been labelled at multiple sites with the fluorophore monobromobimane.

electrophoresis techniques, that is, while the nucleic acid fragments are still embedded within the polyacrylamide gel matrix. 'Post-assay' fluorescent labelling in this manner employs DNA fragments containing multiple phosphorothioate diesters rather than a single phosphorothioate diester, ideally one at every internucleotidic position. The internucleotidic residues are then alkylated with the fluroescent marker monobromobimane.

Monobromobimane has been chosen as the fluorophore for use in post-assay labelling for a number of reasons. It is expected that diffusion of the bimane into the gel matrix readily occurs as a result of its relatively small size. Monobromobimane exhibits acceptable solubility in aqueous or largely aqueous solutions (the polyacrylamide gel matrix is unstable in the presence of many organic solvents). Monobromobimane is essentially non-fluorescent but becomes highly fluorescent after reaction with a thiol or similar derivative. This latter property is important in that the labelled DNA bands will be highly fluorescent while the background of the gel containing unreacted monobromobimane will be largely non-fluorescent.

Protocol 8. Post-assay labelling of phosphorothioate containing DNA in polyacrylamide gels

Materials

- 10% aqueous acetic acid
- Monobromobimane
- Acetonitrile
- Dimethylformamide

Method

1. Prepare a polyacrylamide gel using standard procedures. Add the desired samples and resolve the fragments by electrophoresis.
2. After completion of the electrophoresis, remove the top plate of the gel. If the gel is denaturing, remove the urea by soaking the gel in 10% aqueous acetic acid for 5–10 min.
3. Prepare the monobromobimane solution by dissolving 0.054 g of monobromobimane in 25 ml of acetonitrile, to the mixture add 25 ml of water (final solution is 4 mM monobromobimane in 50% aqueous acetonitrile).
4. Place the monobromobimane solution in a glass dish or try (slightly larger than the gel) and cover the tray with Saran wrap and then with aluminium foil, or store the solution in the dark.
5. After soaking in 10% aqueous acetic acid, transfer the gel into the 4 mM monobromobimane solution, cover with Saran wrap and aluminum foil, and store at ambient temperature overnight.

Protocol 8. *Continued*

6. Destain the gel briefly by gently shaking it in solutions of 50% aqueous acetonitrile (3 × 15 min). The gel may be somewhat cloudy at this point, a brief treatment of 50% aqueous dimethylformamide (1 × 5 min) clears the gel and enhances the fluorescence.
7. Place the gel on a standard long wavelength UV transilluminator (λ_{max} = 366 nm) and photograph using a sharp cut-off filter.

6.3 Detection sensitivity of DNA labelled with multiple fluorophores

The sensitivity of detection (to the naked eye) of DNA fragments labelled in this manner has been determined by the labelling of differing amounts of DNA of varying lengths (or varying numbers of phosphorothioate diesters) and visualizing the fluorescent bands using a standard transilluminator (λ_{max} = 366 nm; the bimane labelled phosphorothioate exhibits an excitation maximum of 390 nm and an emission maximum of 480 nm). Based upon this analysis, an increasing number or bimane fluorophores attached to the DNA fragments results in a corresponding increase in the detection limit. Fragments containing hundreds of potential labelling sites (phosphorothioate diesters) can be visualized in the low femtomolar range (10^{-15} mol) (*Figure 7*) which is very near the sensitivity commonly achieved with radioisotopes.

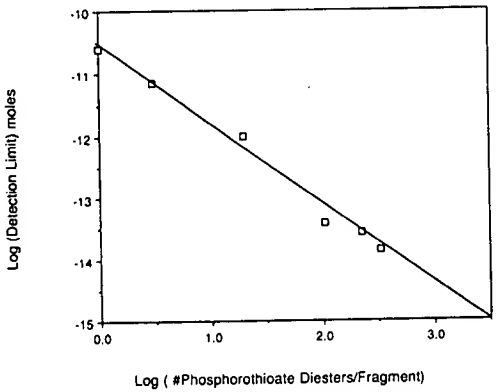


Figure 7. Relationship of detections sensitivity to the number of labelled phosphorothioate diesters.

7. Fluorescent detection of DNA

The multiple labelling procedures described in this section are most applicable to DNA sequencing and hybridization procedures. At present such procedures are not fully developed but would be expected to occur in the following manner.

7.1 DNA sequencing

DNA sequencing using the Sanger sequencing technology employs a DNA template, a primer, the appropriate polymerizing enzyme, the four 2-deoxynucleoside-5′-triphosphates and one 2′,3′-dideoxynucleoside-triphosphate for each of the four reaction mixtures. DNA fragments amenable to post-assay fluorescent labelling can be prepared if the four triphosphates are substituted by a α-thio-2′-deoxynucleoside-5′-triphosphates. This requires virtually no changes in the standard procedures already developed and generates a series of DNA fragments with phosphorothioate diesters at each and every internucleotidic position. After resolution of these fragments by polyacrylamide gel electrophoresis they can be post-assay labelled with the fluorophore monobromobimane.

7.2 DNA hybridization

In a related approach, a DNA hybridization probe can be prepared chemically or enzymatically such that each internucleotidic residue is a phosphorothioate diester. After transfer of the target DNA sequence to the hybridization membrane the hybridization probe can be added using essentially standard procedures. After removal of the excess probe by washing, the hybridization complex can be labelled with monobromobimane using procedures which are analogous to those described for post-assay labelling in polyacrylamide gels.

8. Conclusions

The alkylation of phosphorothioate diesters provides a simple yet versatile technique for the introduction of labels, or potentially agents of any kind, into the DNA backbone. This procedure does not require the synthesis of modified nucleosides nor the preparation of any unique linkers, but rather relies upon standard DNA synthesis techniques which have been modified in order to incorporate phosphorothioate diesters in place of phosphate diesters. Although the alkylation of an internucleotidic phosphorus residue can introduce unwanted additional chirality into the molecule, specific isomers can be obtained if the corresponding phosphorothioate diastereoisomer is prepared. The triesters formed by the described procedures hydrolyze in

alkaline solution but exhibit acceptable stability in acidic, neutral or mildly basic solutions to be valuable for a variety of biochemical and biophysical experiments.

Acknowledgements

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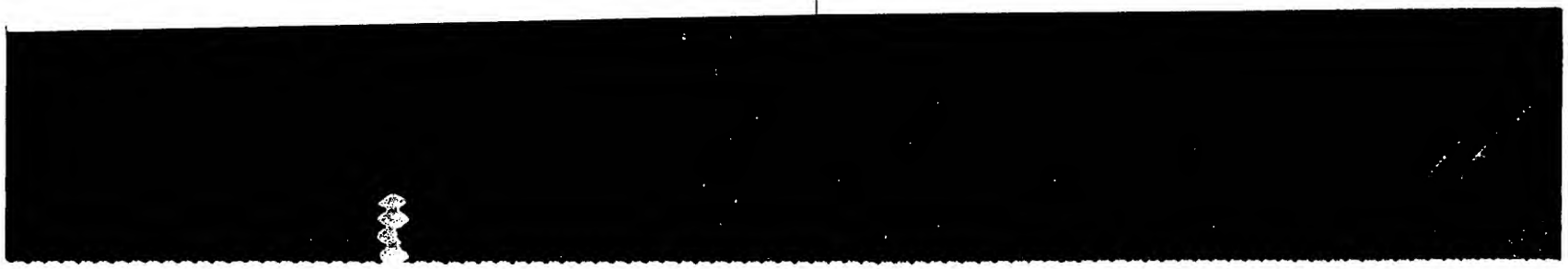


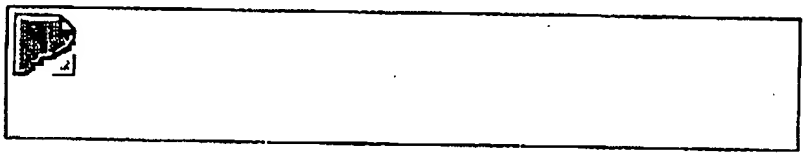
TaqMan® probes

These probes are designed for use with PE Biosystems ABI sequence detection systems. The assay employs a fluorogenic probe that consists of an oligonucleotide with both a reporter and a quencher dye attached at the 5' and 3' end, respectively. The reporter dye options are VIC™, 6-FAM, or TET, whereas, the quencher dye is TAMRA. TaqMan® probes are supplied in three quantities:

TaqMan® probe. HPLC purified approximately 5,000 pmol.
TaqMan® probe. HPLC purified approximately 15,000 to 25,000 pmol.
TaqMan® probe. HPLC purified approximately 50,000 to 100,00 pmol.

Please check with your local sales office for exact specification and price.

- For Research Use Only. Not for use in diagnostic procedures.



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6-FAM, HEX, and TET Phosphoramidites for Fluorescently Labeled Oligonucleotides

Abstract

Three dye phosphoramidites ? [6-FAM], [HEX] and [TET] ? enable direct 5'-end labeling of primers and probes at coupling efficiencies greater than 90%. Oligonucleotides labeled in this manner eliminate hazards associated with the use, storage and disposal of radioactive compounds. The dyes are designed to facilitate analysis of fluorescently labeled PCR products on the ABI PRISM(tm) DNA Sequencer. However, they are equally useful in many applications historically employing radioactivity, such as *in situ* hybridization, cell uptake studies, and target sequence identification.

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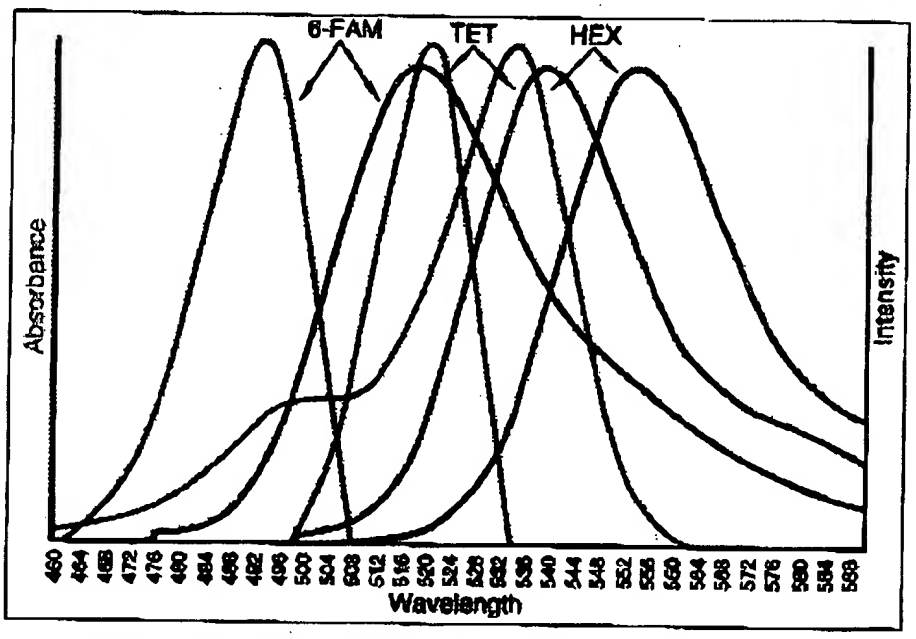

Figure 1. The spectra of [6-FAM]-, [HEX]- and [TET]-labeled oligonucleotides.

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